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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CU01/00003	07/06/2001	07/06/2000

International Patent Classification (IPC) or national classification and IPC
G01N27/447

Applicant
CENTRO NACIONAL DE INVESTIGACIONES CIENTIFICAS

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 11 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08/01/2002	25.07.2002
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Müller, T Telephone No. +49 89 2399 2285

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CU01/00003

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-63 as originally filed

Claims, No.:

1-40 as received on 28/06/2002 with letter of 27/06/2002

Drawings, sheets:

1/18-18/18 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-40
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-40
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-40
	No:	Claims

2. Citations and explanations
see separate sheet

D1: EP-A-0 745 844 (CENT NAC INVESTIG SCIENT) 4 Diciembre 1996 (1996-12-04)
D2: EP-A-0356187, claiming the same priority as ES-A-2 147 174 (THE BOARD OF TRUSTEES OF THE LELAND STRANFORD JUNIOR UNIVERSITY) 1 Septiembre 2000 (2000-09-01) cited in the search report
D5: WO 01 07150 A (KAHL ET AL) 1 Febrero 2001 (2001-02-01)

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Technical field:
The invention is related to a chamber for pulsed field electrophoresis.

2. Prior art:
Closest prior art document D1, cited in the description of the application, discloses miniaturized electrophoresis chambers for transversal alternating filed electrophoresis and for contour clamped homogeneous field electrophoresis systems whose sized are calculated based on the existence of other equipments of larger dimensions.

D1 discloses an electrophoresis chamber (31), and opposed electrodes (32), (33), (34) and (35). The electrode pairs (32) - (35) and (33) - (34) generate a pair of homogeneous electric fields. The relative position of the electrode pair (32) and (35) determines a zone of homogeneous electric field corresponding to the first electric field. The relative position of the electrode pair (33) and (34) determines a zone of homogeneous electric field corresponding to the second electric field. The active zone is localized at the intersection of the two fields. A gel (36) in placed transversely in the zone of homogeneous electric field (see for example page 5, lines 10-15 and figure 3).

3. Novelty:
The following structural features of claim 1 differ from the prior art according to document D1 of the search report:

iii) blocks of materials of high dielectric constant
iv) a fixation and tension system for electrodes

Therefore, the subject-matter of claim 1 is new over the prior art according to D1 (Article 33(2) PCT).

4. Inventive step:
All documents cited in the search report are silent on features iii) and iv) mentioned above. Therefore, the subject-matter of claim 1 is not derivable from the prior art according to the search report, even when combining the disclosure of the available documents. As a consequence the subject-matter of claim 1 does meet the requirements of inventive step 33(3) PCT.

Claims 2-40 are dependent claims and would also meet the requirements of novelty and inventive step (Articles 33(2) and 33(3) PCT)

5. Industrial applicability:
Industrial applicability is given.

6. Remarks:
Documents D2 and D5 were cited for information only. D2 and D5 are categorized as "P"-type documents which means that they could be relevant in the regional phase in case the priority of the application was not valid.

Some of the features in the dependent apparatus claims 22, and 34-40 seem to relate to a method of using the apparatus rather than clearly defining the apparatus in terms of its technical features.

CLAIMS

1.- Pulsed field electrophoresis chambers **with** TAFE (Transversal Alternating Field Electrophoresis) or CHEF (Contour Clamped Homogeneous Electric Field) **electrode arrays** for separating DNA molecules **loaded in gels** by means of using a system for energizing their electrodes and alternating the direction of application of the electric field **generated by the electrode array**, as well as a system for circulating the buffer, which chambers comprise:

i) A minigel, or various minigels placed in the zones that are crossed by the lines of force of the electric field that directly interact with the molecules loaded into said minigel(s); zones which are the useful electrophoresis zones (UEZ) of the chamber,

ii) pairs of electrodes of opposite polarities separated in the electrode array a distance 'd', which is from 6.2 to about 15 cm, separation 'd', which in conjunction with the number and sizes of UEZs limit the height, depth and width of the chamber to certain values, and also limit the minigel sizes and the total number of samples that can be loaded simultaneously in all minigel(s) placed into said chamber;

iii) Blocks of materials of high dielectric constant occluding the zones of said chambers that are crossed by the electric field force lines that do not act on the molecules loaded in the minigel(s); zones which are the non-useful electrophoresis zones (NEZ) of the chamber,

iv) stretched electrodes, that are pulled tight by the action of a fixation system in CHEF chambers and by a fixation and tension system in TAFE chambers,

v) electrode array(s) of TAFE system that have the anode at the bottom of the chamber and the cathodes at the top of the chamber (inverted TAFE electrode configuration);

vi) three accessory sets of said CHEF and TAFE chambers whereby the flow of electric current through the chambers is homogenized; the first set is formed by removable rectangular sheets that occupy parts of said chambers, sheets whereby the buffer is circulate at high flow velocity, the second one comprises disassemblable devices formed by frames, base plates, covers and combs, devices whereby minigels of said chambers are cast with homogeneous cross area, and the third one comprises disassemblable systems of blocks, covers, and cutters whereby the sample miniplugs of said minigels are cast;

being said CHEF and TAFE chambers and their accessory sets completely assembled and used according to specific methods of use, chambers in which the separations of DNA molecules are done according to methods of performing the electrophoresis in said chambers, methods of use which comprise several steps.

2.- Electrophoresis chambers as claimed in claim 1 wherein the CHEF chambers have single UEZ that can support a rectangular or square shaped minigel.

3.- Electrophoresis chambers as claimed in claim 2 wherein the rectangular-shaped minigel of CHEF chambers is d/3 cm in length and d/1.732 cm in width (a), being the width from 3.6 to about 8.7 cm, and the length from 2.1 to about 5 cm.

4.- Electrophoresis chambers as claimed in claim 2 wherein the length and the width 'a' of the square-shaped mini-gel of CHEF chambers is d/3 cm, being 'a' from 2.1 to about 5 cm.

5.- Electrophoresis chambers as claimed in claim 1 wherein the floor of CHEF chamber comprising the UEZ has an area equals to $[2 +(d/0.87)]\bullet[6 + d]$, being said area from about 111.3 to about 404.1 cm^2, whereas the remaining parts of the floor of said chamber are covered with blocks of materials of high dielectric constant.

6.- Electrophoresis chambers as claimed in claim 1 wherein chambers with TAFE electrode array have a rectangular frontal wall with the largest side parallel to any electrode of the array and up to 50 cm in length ('L'), chamber that support a minigel in a single UEZ or more minigels in the UEZs formed by dividing the length of the largest side of said wall of the chamber and the electrodes of the array.

7.- Electrophoresis chambers as claimed in claim 1 wherein the length of the minigels of TAFE chambers is $d \bullet 0.515$ cm, length that is from 3.2 to about 7.7 cm.

8.- Electrophoresis chambers as claimed in claim 1 wherein a minigel of CHEF or TAFE chambers has 'N' wells that support 'N' as the maximum number of miniplugs, being N equal to (a – 0.2) / 0.25, and 'a' the width of minigel, which is from 1.7 to 50 cm in TAFE.

9.- Electrophoresis chambers as claimed in claim 1 wherein the area corresponding to the UEZ in the side walls of TAFE chamber, or walls that support the gel and the electrodes, is equal to $[2+1.4 \bullet d] \bullet [2 + 0.54 \bullet d] - 1.02 \bullet [1+ 0.54 \bullet d]^2$, being said area from 37.8 to about 149.5 cm^2, whereas the parts of said side walls corresponding to the NEZ are blocked with pieces of high dielectric constant.

10.- Electrophoresis chambers as claimed in claim 1 wherein TAFE chambers are evenly subdivided in several UEZs with a minigel each one, minigels that are placed widthwise in tandem, sequentially one next to the other, with their faces parallel to the electrodes.

11.- Electrophoresis chambers as claimed in claim 1 wherein TAFE chambers have fixed or removable single electrode platform that contains the electrode array (type I TAFE chamber) being the length 'L' of said electrodes up to 50 cm.

12.- Electrophoresis chambers as claimed in claims 1 and 11 wherein the single electrode platform of type I TAFE chamber is evenly subdivided and forms several UEZs with all minigels supported in a single frame.

13.- Electrophoresis chambers as claimed in claims 1 and 11 wherein the single electrode platform of type I TAFE chamber is evenly subdivided and forms several UEZs with each minigel independently placed in an UEZ, for which said chambers must have laterally grooved pieces to slide said minigels.

14.- Electrophoresis chambers as claimed in claim 1 wherein a TAFE chamber has various fixed or removable independent mini-platforms of electrode array with a minigel each one, platforms whereby the useful electrophoresis zone (UEZ) are limited, and comprising electrodes physically separated from the electrodes of the remaining platforms of said chamber, but able to be plugged in parallel with them to acquire continuity; so, when the chamber is energized with a single power supply, all samples loaded in the minigels of the platforms are at the same electrophoresis conditions (type II TAFE chamber).

15.- Electrophoresis chambers as claimed in claim 1 wherein a TAFE chamber has pieces of the proper shape made of any material with high dielectric constant, pieces that occupy and fully occlude the regions of the chamber corresponding to the useful electrophoresis zones (UEZs) and are as many as required to analyze the desired number of samples in the minimal amount of UEZs.

16.- Electrophoresis chambers as claimed in claim 1 wherein TAFE chambers have from 1 to 30 UEZs and support from 1 to 30 minigels.

17.- Electrophoresis chambers as claimed in claim 1 wherein said TAFE chambers can be in inverted TAFE configuration, configuration that has the cathodes of the miniplatforms at the bottom of the electrophoresis chamber and the anodes at the top, thus being the samples loaded in the minigel bottom, so, the samples migrate in the direction opposite to the gravity.

18.- Electrophoresis chambers as claimed in claim 1 wherein TAFE chambers have either external walls parallel to the imaginary plane containing the cathode of one electric field and the anode of the other electric field, being these walls the ones that do not support the electrodes, and being they placed at most 2 cm apart from said imaginary plane, or blocks made of materials with high dielectric constant that occupy the parts of the chamber corresponding to the non useful electrophoresis zones (NEZ).

19.- Electrophoresis chambers as claimed in claim 1 wherein the electrodes, which are kept fixed by the action of a fixation system in CHEF and TAFE chambers, enter into the chamber from the outside, are energized with a single power supply during the electrophoresis and enter in contact with the buffer passing through the bores of elastic plugs inserted into holes drilled in the floor of CHEF chambers or in the walls of TAFE chambers supporting the gel, said plugs being used to fix the electrodes to the chamber.

20.- Electrophoresis chambers as claimed in claims 1 and 19 wherein the elastic plugs through which the electrodes pass can be made of silicone, rubber or any other elastic material.

21.- Electrophoresis chambers as claimed in claim 1 wherein TAFE chambers have a system to pull tight the electrodes crossing the walls of said chamber, system which is placed at the exit of each electrode, being said system comprised of:

i) a rod that is slotted in its top side, rod that is able to turn and has a waist-shaped notch crossed by a hole into which the end of the electrode is inserted and bent around the rod waist,

ii) a grub screw which sets definitely the rod in the desired position.

22.- Electrophoresis chambers as claimed in claims 1 and 21 wherein the method of use of the fixation and tension system of the electrodes of TAFE chambers comprises the following steps:

i) loosening the grub screw that fixes the rod into which the electrode is inserted,

ii) turning the rod the required angle for pulling tight said electrodes,

iii) tightening the grub screw to set the rod in the position that maintains the electrode stretched.

23.- Electrophoresis chambers as claimed in claim 1 wherein the set of removable rectangular sheets of CHEF chambers has two types of rectangular sheets: the 'A' and the

'B' types, both made of a material with high dielectric constant, sheets whose largest sides are as wide as the chamber width, and the other sides are at least 2 cm in height in the 'A' type sheet and 0.5 cm in the 'B' type.

24.- Electrophoresis chambers as claimed in claims 1 and 23 wherein the 'A' type sheets are placed over the chamber floor protruding from the surface of the buffer solution, sheets over the floor that are separated from it from 0.02 to about 0.05 cm and form a gap between their inferior edges and the floor, gap throughout the buffer solution can flow only.

25.- Electrophoresis chambers as claimed in claims 1 and 23 wherein the B type sheets are glued to the floor of the chamber and fully submerged into the buffer, so circulating buffer only flows over the 'B' type sheets.

26.- Electrophoresis chambers as claimed in claims 1 and 23 wherein both types of sheets (A and B) are arranged alternately from the buffer inlet and outlet toward the electrode array, arrangement made in the following order: an A type sheet followed by a B type sheet, repeating 'n' times said pair of sheets, being 'n' an integer between 1 and 4, and placing the last sheet about 1 cm apart from the electrodes and being said last sheet of A type.

27.- Electrophoresis chambers as claimed in claim 1 wherein the set of removable rectangular sheets of TAFE chambers are two identical sheets made of a material with high dielectric constant, sheets similar to the walls of the chamber and placed in parallel with the plane that contains the electrodes of the same gel side, being said sheets horizontally slotted up to 0.5 cm in their inferior third, and being the slot as large as the chamber or minigel width.

28.- Electrophoresis chambers as claimed in claims 1 and 27 wherein the two removable sheets of TAFE chambers are placed as follows: one near to the buffer inlet and the other

near to the outlet, sheets that divide the chamber in three compartments: the central one, containing the UEZ, and two lateral ones through which the buffer is delivered into the chamber or is withdrawn from it.

29.- Electrophoresis chambers as claimed in claim 1 wherein the set of disassemblable devices to cast minigels, devices formed by frames, base plates, covers and several comb-shaped pieces with teeth of identical width and identical thickness each one, devices which are comprised by:

i) a flat base plate,

ii) two frames with two notches for inserting the combs, frames from 0.35 to about 0.5 cm in thickness with rectangular or square shaped cavities that determine the shape, thickness, length and width 'a' of the minigels cast in them; minigels which are the supporting medium of the electrophoresis in CHEF or TAFE chambers,

iii) a comb with long teeth whereby wells are formed in the minigel; wells where sample miniplugs are loaded,

iv) two covers: the cover 1 that fits against the front of the comb, and the cover 2 that fits against the back of the comb,

v) another comb, similar to the comb with long teeth, but with shorter teeth, comb whereby sample miniplugs are pushed and aligned into the minigel wells.

30.- Electrophoresis chambers as claimed in claims 1 and 29 wherein the comb with long teeth is flat in its frontal part, whereas in the rear and over the teeth it is thicker forming a step, comb with teeth of identical sizes, which are: from 0.03 to about 0.1 cm in thickness, from 0.15 cm up to the minigel width 'a' minus 0.3 cm in width (a – 0.3), and length equal to the minigel thickness (th) minus 0.1 cm (th - 0.1).

31.- Electrophoresis chambers as claimed in claims 1 and 29 wherein the comb with short teeth has shape and sizes similar to the comb with long teeth, excepting the length of the teeth, which are about 0.2 cm shorter.

32.- Electrophoresis chambers as claimed in claims 1 and 29 wherein the cover 2, or cover fitting against the rear of the comb, has two flat surfaces and a protruding edge, whereas the cover 1, fitting against the front of the comb, has two flat surfaces but one of its edges has a bevel cut in wedge formation.

33.- Electrophoresis chambers as claimed in claim 1 wherein the set of disassemblable system of blocks, covers and cutters to form the sample miniplugs of said minigels is comprised of:

i) various sample plug makers, each one composed by a flat impermeable block, thicker than 0.5 cm, block that has several parallel grooves lengthwise, being the width of each groove 0.2 cm, and the depth equal to the thickness of the teeth of a given comb, being said depth from 0.03 to about 0.1 cm, and existing plug makers for all possible teeth thickness of the combs with long teeth that can be used to form the minigel wells,

ii) a flat rigid and impermeable sheet of at least 0.1 cm in thickness, which acts as the cover of the sample plug block,

iii) several sample plugs cutters, each being a bar which is as long as or longer than the grooves of the block of the sample plugs maker, said cutters having legs in the ends which confer them an inverted-U shape, said cutters having several protuberances with cutting edges in its inferior part, said protuberances protruding 0.1 cm from the bar, said cutting edges being transversal to the longest dimension of the bar and 0.2 cm in length, said cutting edges being evenly spaced a distance that is from about 0.15 to the gel width minus 0.3 cm.

34.- Electrophoresis chambers as claimed in claim 1 wherein the method of use of the disassemblable system of blocks covers and cutters to form the sample miniplugs with sizes (depth, width and thickness) similar to the sizes of the wells of the minigel comprises the following steps:

i) preparing a cell suspension in molten agarose and keeping it at 45 °C,

ii) pre-warming the grooved block, of the sample plug maker, and its cover at 45 °C,

iii) pouring said suspension in the grooves of the block,

iv) covering the grooved block with its cover-plate and maintaining the set at room temperature or at lower temperature until the agarose solidifies,

v) aligning the sample plugs cutter lengthwise on the first groove of the block with the protruding cutting edges turned downward,

vi) pressing down the sample plug cutter and further removing it from the set,

vii) tilting the grooved block and pushing the sample plugs into a vessel containing the proper solution for their treatment,

viii) repeating the process for all agarose strips solidified in all grooves of the block.

35.- Electrophoresis chambers as claimed in claim 1 wherein the method of use of the disassemblable device formed by frames, base plates, covers and combs whereby the minigels of the UEZ of said chamber are cast with homogeneous transversal area comprises the following steps:

i) placing the frame on the flat base plate,

ii) fitting the legs of the comb with long teeth into the notches of the frame, or notches milled in the outer sides of the frame,

iii) placing the cover 1 on the frame and in front of the comb, with the flat surface turned to face the frame, the bevel edge against the comb,

iv) clamping the set until the interstices are sealed,

v) maintaining the molten gel between 65 and 70 °C,

vi) pouring the molten gel into the cavity, filling the cavity formed between the frame, the flat base plate and the cover 1,

vii) placing the cover 2 on the frame, introducing the protruding edge of the cover into the rear step of the comb with long teeth, thus eliminating the excess of molten agarose,

viii) leaving the system to set until the gel is solidified,

ix) removing the comb with long teeth, leaving the wells of the desired width and thickness formed in the gel,

x) placing the sample plugs on the wedge-shaped edge of the cover 1 and pushing said plugs with an applicator to slide them into the wells,

xi) placing the comb with short teeth in the set, by fitting into the notches of the frame the legs of said comb, then pushing said sample plugs to the bottom of the wells,

xii) removing the cover 1, the cover 2 and the frame from the set.

36.- Electrophoresis chambers as claimed in claim 1 wherein the method to perform the electrophoresis in TAFE chambers that have various UEZs for analyzing 'x' number of samples comprises the following steps:

i) defining the minimum number of UEZ (or minigels) required to load the 'x' number of samples (x is an integer) in the chamber,

ii) occluding the UEZs not required in the electrophoresis to analyze the 'x' number of sample,

iii) plugging in parallel the electrodes of the UEZs that will be energized (activated), if TAFE chamber with various electrode platforms (type II chamber) will be used.

37.- Electrophoresis chambers as claimed in claim 1 wherein said CHEF chambers require to be filled with buffer solution up to a level that surpasses the gel thickness by at least 0.3 cm to perform the electrophoresis in it, being it accomplished by adding buffer to the chamber, buffer volume that is calculated from the knowledge of 'd', or separation between the electrodes with opposite polarity in the array, according to the formula $\{[2 + (d / \cos(30^{\circ}))] \bullet [6 + d]\} \bullet (0.3 + \text{gel thickness})$, being the volume added to the chamber from about 72.3 to about 323.3 ml of if the gels are from 0.35 to 0.5 cm thickness.

38.- Electrophoresis chambers as claimed in claim 1 wherein said TAFE chambers require to be filled with buffer solution up to a level that surpasses the gel height by at least 0.3 cm to perform the electrophoresis in it, being it accomplished by adding buffer to the chamber, volume that can be calculated from the knowledge of 'd', or separation between the electrodes with opposite polarity in the array, and the number of active UEZs in the chamber ($NZUE_{active}$) according to the formula $[(2 + 1.4 \bullet d) \bullet (2 + 0.54 \bullet d) - 1.02 \bullet (1 + 0.54 \bullet d)^2] \bullet L \bullet NZUE_{active}/NZUE_{total}$, being said volumes from about 63.2 to about 7390 ml.

39.- Electrophoresis chambers as claimed in claim 1 wherein CHEF chambers and the TAFE chambers of single active UEZ admit to be energized at electric field strengths up to 16 or 25 V/cm, respectively, provided the chambers are energized using power supplies with a maximum power output of 300 watt and the buffer solution is maintained at constant temperature, being it from about 4 to about 30 °C.

40.- Electrophoresis chambers as claimed in claims 1 and 36 wherein the TAFE chambers of several active UEZ admit to be energized at electric field strength from 8 to 25 V/cm, electric field that depends on the number of UEZ activated, provided the buffer is maintained at constant temperature, being it from 4 to 30 °C.